Award Number:

W81XWH-10-1-0764

TITLE:

Priming the tumor immune microenvironment improves immune surveillance of cancer stem cells and prevents cancer recurrence.

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REPORT DATE:

October 2012

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
1 October 2012	Annual	9/15/2011 – 9/14/2012

4. TITLE AND SUBTITLE
Priming the tumor immune microenvironment improves immune Surveillance of cancer stem cells and prevents cancer recurrence

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0764

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Scripps Research
Institute
La Jolla, Ca 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release;
Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The cancer stem cell (CSC) hypothesis has gained significant recognition as a descriptor of tumorigenesis. Additionally, tumor-associated macrophages (TAMs) are known to promote growth and metastasis of breast cancer. However, it is not known whether TAMs mediate tumorigenesis through regulation of breast CSCs. Here, we report that TAMs promote CSC-like phenotypes in murine breast cancer cells by upregulating their expression of Sox-2. These CSC-like phenotypes were characterized by increased Sox-2, Oct-4, Nanog, AbcG2 and Sca-1 gene expression, in addition to increased drug–efflux capacity, resistance to chemotherapy and increased tumorigenicity *in vivo*. Downregulation of Sox-2 in tumor cells by siRNA blocked the ability of TAMs to induce these CSC-like phenotypes and inhibited tumor growth *in vivo*. Furthermore, we identified a novel EGFR/Stat3/Sox-2 paracrine signaling pathway between macrophages and mouse breast cancer cells that is required for macrophage-induced upregulation of Sox-2 and CSC phenotypes in tumor cells. We showed that this cross-talk was effectively blocked by the small molecule inhibitors AG1478 or CDDO-Im against EGFR and Stat3, respectively. Therefore, our report identifies a novel role for TAMs in breast CSC regulation and establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for cancer stem cell therapy.

15. SUBJECT TERMS
Sox-2 upregulates CSC phenotype; TAMs mediates tumorigenesis; Sox-2 signaling is controlled by Stat-3; EGRF/Sox-2 signlaing is a therapy target

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	11	USAMRMC

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INTRODUCTION:

The cancer stem cell hypothesis postulates that neoplastic clones are maintained by a small subpopulation of cells that possess the capacity for self-renewal and differentiation potential, thus giving rise to cancer cells that comprise the tumor bulk. Furthermore, cancer stem cells (CSCs) have been suggested to be the root cause of cancer recurrence and disease relapse due to their resistance to chemo- and radiotherapy. Another useful approach for identifying CSCs, particularly in the absence of suitable surface marker expression, utilizes the phenomenon of stem cells' unique ability to efflux lipophilic fluorescent dyes, including HOESCHT 33342. This efflux capacity was shown to correlate with expression of ABC transporters and could be specifically inhibited with Ca++-channel blockers. The dye-effluxing population was given the designation SP based on their low dye retention characteristic. Tumor associated macrophages (TAMs) constitute a major cell population in the breast TME. Importantly, it has been suggested that macrophages secrete growth and other factors that permeate the breast stem cell niche to promote survival and self-renewal of stem cells.

BODY:

1. TAM-associated increases in breast cancer tumorigenicity correlate with increased percentage of SP cells *in vivo*. Interactions between CSCs and cells within their niche in the tumor microenvironment (TME) appear to be important for their maintenance and function. The impact of TAMs on breast CSC maintenance and tumorigenicity *in vivo* was assessed with isolated 4T07 SP cells by flow cytometry and injection of these cells into cleared fat pads of syngeneic Balb/c mice that were either depleted or not of macrophages by clodronate liposomes (Figure 1A). Depletion of macrophages in both blood and tumor tissue was confirmed by flow cytometry (Figure 1B). Analysis of mice 25 d after tumor cell implantation revealed significantly reduced primary tumor and lung weights in animals treated with clodronate liposomes, indicating reduced tumorigenicity and metastatic potential, respectively, in mice depleted of macrophages (Figures 1 C and D). Reduction in tumorigenicity and metastasis in macrophage-depleted mice correlated with a significant decrease in the percentage of HOECHST 33342 dye-effluxing SP cells in primary tumors (Figure 1E).

2. TAM co-culture enriches SP population of 4TO7 tumor cells in vitro. The mechanism of TAM-mediated regulation of breast CSCs was elucidated by a series of *ex vivo* experiments involving co-culture of murine breast cancer cells with the murine RAW 264.7 macrophage cell line. Flow cytometry analysis revealed that co-culture of tumor cells with RAW macrophages resulted in a 2.8-fold increase in percentage of SP cells (Figure 2A), which correlated with a marked increase in expression of Sca-1 and AbcG2 on the surface of these cells (Figure 2B). At the mRNA and protein levels, macrophage co-culture resulted in increased mRNA and protein expression of Sox-2, Oct-4 and Nanog by SP cells (Figures 2 C and D, respectively). Further analysis of the expression of Sox-2, Oct-4 and Nanog in primary tumors by immunohistochemistry revealed that these key transcription factors were expressed by tumor cells proximal to TAMs (Figure 2E-F). Together, these results suggest that TAMs actively communicate

with tumor cells to enhance their expression of Sox-2 and other key regulators of CSC phenotypes. These findings further imply that TAMs regulate breast CSCs by mediating their expression of Sox-2 through paracrine signaling within the stem cell niche.

3. The Sox-2 transcription factor regulates the tumorigenicity of SP cells. Transcriptional regulation plays a key role in maintenance of cancer stem cell properties and the Sox-2 transcription factor was reported important for regulating ES cells. We postulated that the Sox-2 signaling pathway might be involved in networks controlling breast CSC maintenance. Thus, siRNA was used to silence Sox-2 gene expression in 4TO7 cells, which was confirmed by RT-PCR (Figure 3A). Intriguingly, Sox-2 silencing also resulted in decreased Oct-4 and Nanog mRNA expression (Figure 3B). Expression of Sca-1 and AbcG2 stem cell markers was markedly suppressed in the SP population of 4TO7 cells treated with Sox-2 siRNA (Figure 3C) and i*n vivo*, Sox-2 knockdown in 4TO7 cells markedly suppressed tumor growth (Figure 3D), reduced tumor weights (Figure 3E), and decreased lung metastasis, as indicated by decreased lung weight (Figure 3F). *Ex-vivo* transwell migration assays showed that Sox-2 silencing also suppressed tumor cell motility (Figure 3G). Sox-2 silencing in SP cells not only increased tumor cell apoptosis, but also increased the sensitivity of these cells to mitoxantrone chemotherapy (Figure 3H). Together, these data confirm that transcription factor Sox-2 is important for the maintenance of CSC phenotypes in murine breast cancer cells.

4. TAM activation of EGFRs on CSCs increases Sox-2 expression by tumor cells. An effort was made to identify signaling molecules responsible for TAM-mediated upregulation of Sox-2 expression by breast cancer cells. Since aberrant activation of the EGF receptor (EGFR) signaling pathway has been observed in many human cancers, we determine if soluble EGF released by TAMs mediates acquisition of CSC phenotypes by breast cancer cells. EGFR and phosphorylated EGFR (pEGFR) on 4T1 cells co-cultured with RAW macrophages showed by flow cytometry overexpression of both EGFR and pEGFR by SP cells, compared with Non-SP cells, in pure 4T1 cultures (Figure 4A). Expression of EGFR and pEGFR by SP cells correlated with an increase in percentage of SP cells, compared with tumor cells cultured alone (Figure 4D). Culture of tumor cells with recombinant mouse EGF (mEGF) also induced an increase in the percentage of SP cells and a concordant increase in EGFR and pEGFR expression by these cells (Figure 4E). Importantly, mEGF treatment resulted in increased expression of Sox-2 mRNA and protein by 4T1 tumor cells (Figure 4B). This increase in Sox-2 expression also correlated with increased tumor cell migration upon mEGF stimulation (Figure 4C). Together, these results demonstrate that TAM/tumor cell crosstalk via EGF/EGFR functions as an upstream activator of Sox-2 expression in murine breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated a key role for tumor-associated macrophages (TAMs) in promoting

- CSC phenotypes in murine breast cancer cells.
- Identified a novel mechanism of regulation achieved by paracrine EGF signaling between TAMs and breast cancer cells.
- Established that signaling between TAMs and tumor cells involves activation of the EGFR/Stat3 signaling p0athway and the downstream upregulation of transcription factor Sox-2.
- Importantly, crosstalk between TAMs and tumor cells was found to require EGF3 and Stat3 activation that could be blocked by small molecule inhibitors of either EGF or Stat3.
- Our findings identified a novel role for TAMs in breast CSCs regulation and establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for cancer stem cell therapy.

REPORTABLE OUTCOMES:

Poster presentation at AACR Annual Meeting at Chicago, IL in the TB02-05 Cancer Stem Cell session on 4/2/12. The presentation was entitled "An inhibitor of the NF-Kappa B pathway targets cancer stem cells and prevents tumor recurrence".

CONCLUSION:

To our knowledge, we are the first to describe a unique interaction between TAMs and breast cancer cells via EGF/EGFR/Stat3 signaling which is critical for the expression of transcription factor Sox-2 and the maintenance of breast cancer stem cells. Importantly, we identified a novel role for macrophages on the regulation of breast cancer stem cells which establishes a rationale for targeting the EGFR/Stat3/Sox-2 signaling pathway for breast cancer stem cell therapy

APPENDICES:

Figure Legends:

Figure 1 TAMs mediate SP cell maintenance *in vivo*.

(A) 4TO7-SP cells were isolated by HOECHST 33342 dye staining and flow cytometry cell sorting. These SP cells ($1x10^3$) were injected i.v. into Balb/c mice that had previously been depleted of macrophages by treatment with chlordonate liposome nanoparticles (M φ KO). Control animals were treated with saline and thus not depleted of macrophages (WT). (n=5 mice/group), (B) Macrophage populations ($CD45^+/F4/80^+$) in blood and primary tumors of WT or M φ KO mice were measured by flow cytometry. Data represent means±S.E.M. Mice were sacrificed 25 d after SP cell challenge and tumor (C) and lung (D) weights were measured. Data represent means±S.E.M. (E) Percentages of SP cells in primary tumors from WT and M φ KO mice were measured by HOECHST 33342 dye staining and flow cytometry. Data represent means±S.E.M. *$p<0.05$, **$p<0.005$.

Figure 2 TAMs enrich SP cells and enhance expression of Sox-2, Oct-4 and Nanog in breast cancer cells.

(A) SP of 4T07 breast cancer cells were obtained by HOECHST staining and Flow cytometry after 96 h of co-culture with either TAMs derived from 4T07 tumor tissue or RAW macrophages. (B) Expression of Sca-1 and ABCG2 was also detected in this same population. (C) Expression of Sox-2, Oct 4 and Nanog was determined by RT-PCR, and (D) Western blot. (E, F) Expression of Sox-2, Oct 4 and Nanog in 4T07 tumor tissue was confirmed by Immunofluorescence histology staining. Scale bars, 100μm; 150 μm on lower panel of E.

Figure 3 Transcription factor Sox-2 regulates maintenance of cancer stem cell-like properties of SP cells.

Sox-2 gene expression in 4TO7 cells was silenced by siRNA. (A) Down regulation of Sox-2 was confirmed by RT-PCR. (B) Gene expression of Sox-2, Oct-4 and Nanog by SP cells was determined by RT-PCR after Sox-2 silencing. (C) Expression of surface makers Sca-1 and ABCG2 was assessed by flow cytometry in 4TO7 SP cells after Sox-2 silencing. (D) Balb/c mice, challenged with 4TO7-SP wild type (WT) or 4TO7-SP cells were subjected to Sox-2 silencing (Sox-2 siRNA), and tumor volumes measured every 3-4 d. (n=5mice/group). 25 d after tumor cell challenge, tumor (E) and lung (F) weights were measured. Data represent means±S.E.M. (G) Migration assays were performed on 4TO7 WT or Sox-2 siRNA-treated SP cells with Boyden transwell chambers. (n=3 wells/group). (H) Effects of Sox-2 silencing on apoptosis of Non-SP and SP cells were determined by Annexin V staining and flow cytometry. Sensitivity of SP and Non-SP cells to mitoxantrone chemotherapy was assessed after Sox-2 knockdown.

Figure 4. TAMs and EGF induce overexpression of EGFR and pEGFR on SP cells that correlates with increased Sox-2 expression and cell motility, which are inhibited by EGF neutralizing antibody.

A) The expression of EGF in F4-80+ cells from either 4T1 tumor tissue or normal spleen of Balb/c mice was determined by Flow cytometry. EGF expression in RAW cells after co-cultured with 4T1 tumor cells was also evaluated simultaneously. (B) Expression of Sox-2 at the mRNA (left panels) and protein levels (right panels) in 4T1 cells was assessed after treatment with mEGF. (C) EGF-induced migration of 4T1 cells as determined by using Boyden transwell chambers. Expression of EGFR or phosphorylated EGFR (pEGFR) was detected by HOECHST 33342 dye and antibody staining, followed by flow cytometry analysis of 4T1 SP and Non-SP cells after co-culture with RAW macrophages (D) or recombinant mouse EGF (mEGF) (E). Data represent means±S.E.M.

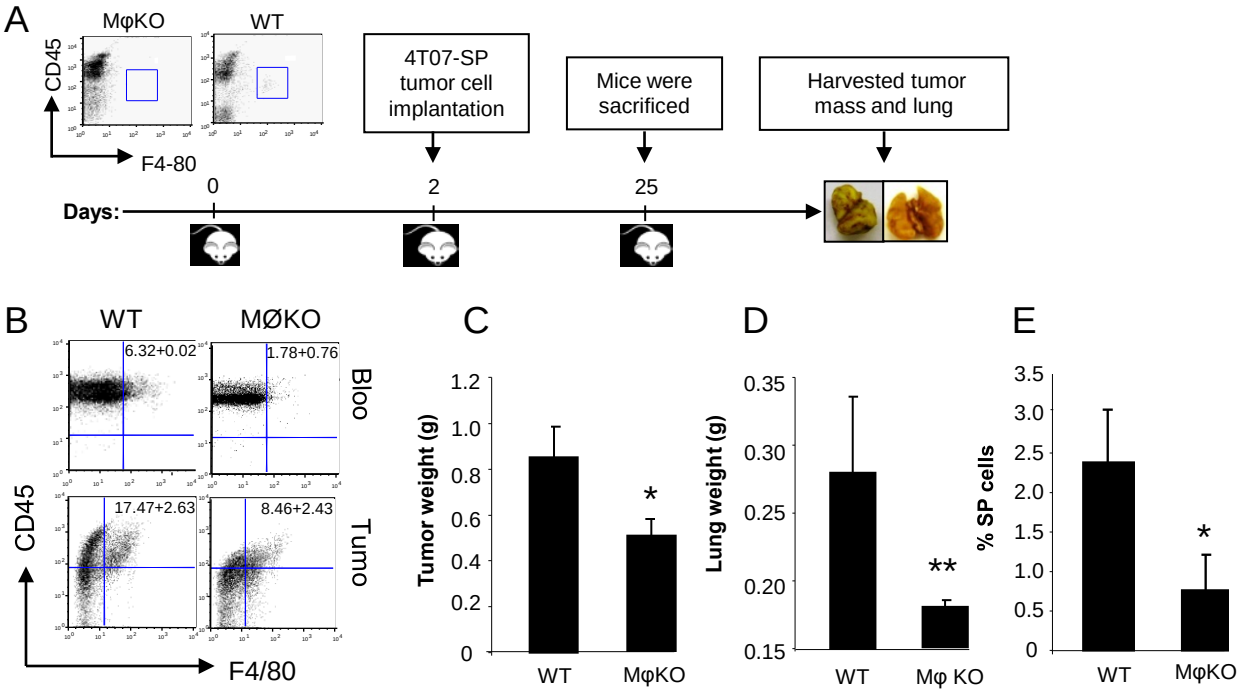
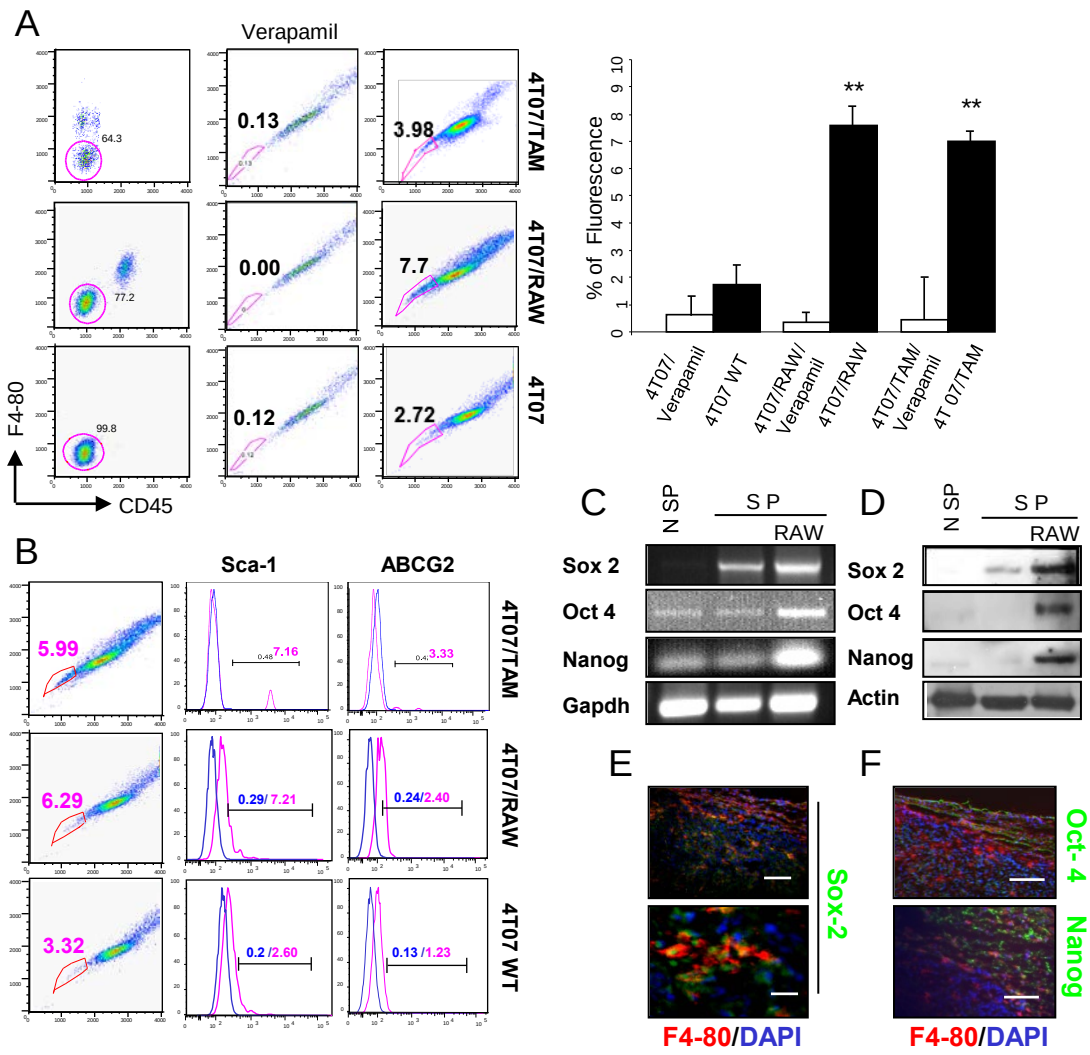


Figure 1. TAMs mediate SP cell maintenance *in vivo*.



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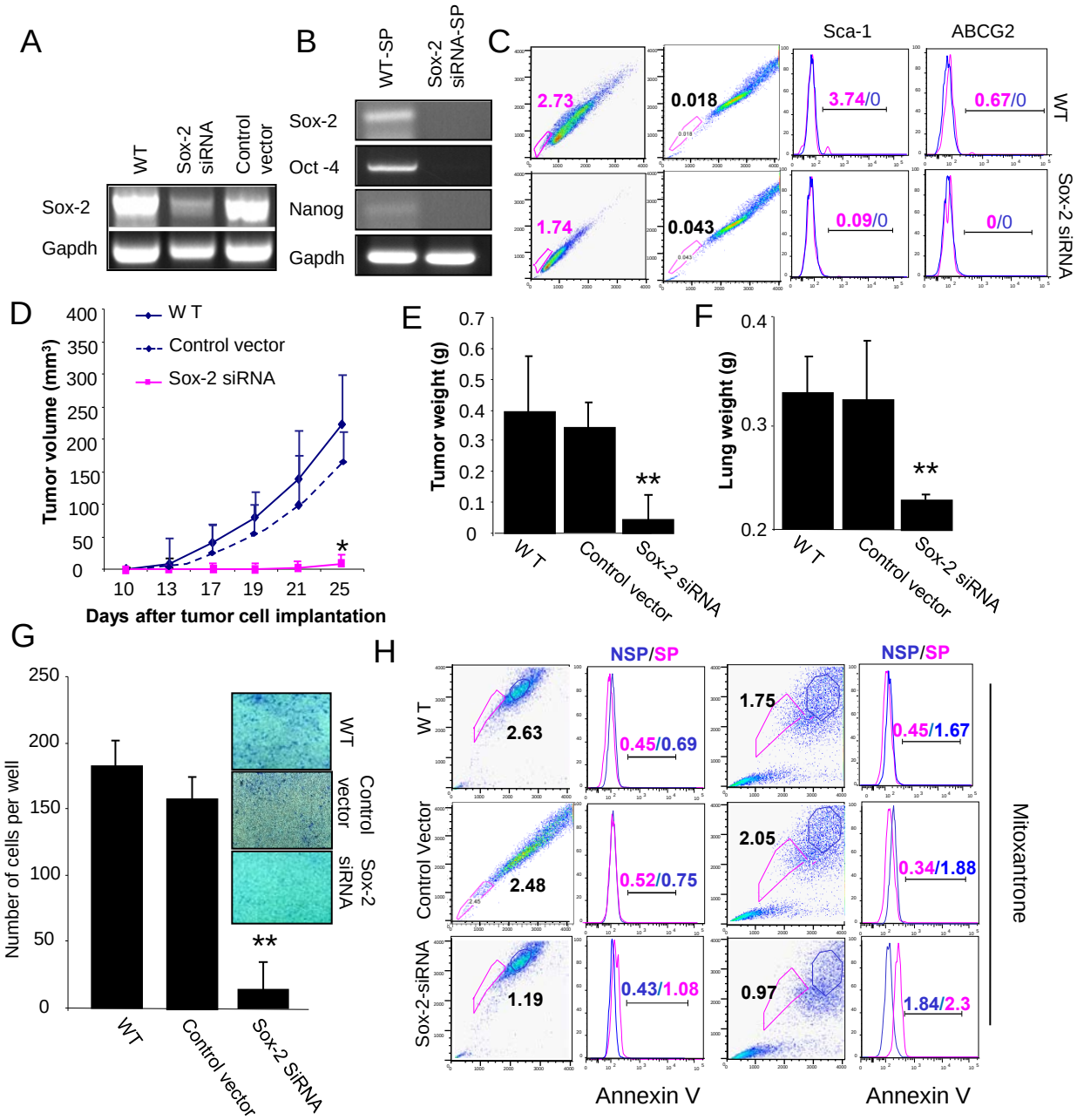
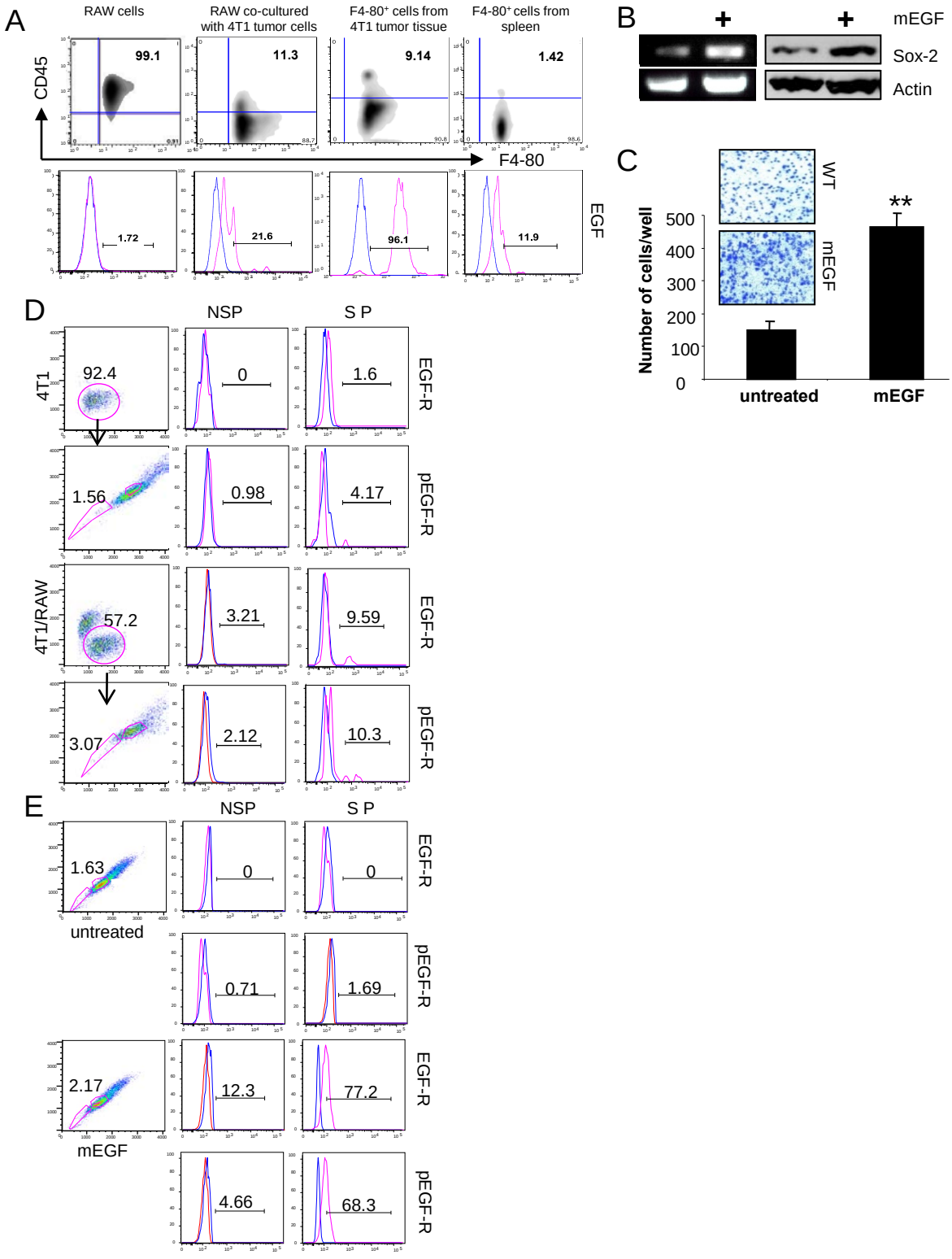


Figure 3. Transcription factor Sox-2 regulates the maintenance of cancer stem cell-like properties of SP cells.



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